CONTROL OF APOPTOSIS

The present invention relates to the control of programmed cell death, apoptosis, and related gene expression and, in particular, it relates to methods of, and means for, controlling apoptosis in cells and modulating, preferably suppressing, the expression of a particular, selected apoptosis-related gene.

The ability to selectively suppress the expression of a gene is useful in many areas of biology, for example in methods of treatment where the expression of the gene may be undesirable; in preparing models of disease where lack of expression of a particular gene is associated with the disease; in modifying the phenotype in order to produce desirable properties. Thus, the ability to selectively suppress the expression of a gene may allow the "knockout" of human genes in human cells (whether wild type or mutant) and the knockout of eukaryotic genes in studies of development and differentiation.

The ability to selectively induce cell death is important in targeting and destroying tumour cells in cancers, as well as in other situations, for example in the resolution of inflammation. It is useful in directly killing the cells which have become oncogenic, or other cells in the tumour (such as leukocytes, for example macrophages), as well as in enhancing any other therapies, since tumour resistance to cancer therapies is a major problem in treatment of the disease. Apoptosis is one of the major mechanisms for inducing cell death and resistance to apoptosis is a naturally acquired characteristic during tumour progression. This usually involves altered

regulation of apoptotic signalling molecules that render tumor cells unresponsive to apoptotic stimuli.

Methods of attempting to suppress the expression of a particular gene fall mainly into three main categories, namely antisense technology, ribozyme technology and targeted gene deletion brought about by homologous recombination.

Antisense techniques rely on the introduction of a nucleic acid molecule into a cell which typically is complementary to a mRNA expressed by the selected gene. The antisense molecule typically suppresses translation of the mRNA molecule and prevents the expression of the polypeptide encoded by the gene, whilst the antisense molecule remains bound to the mRNA molecule. Modifications of the antisense technique may prevent the transcription of the selected gene by the antisense molecule (triplex forming oligonucleotide; TFO) binding to the gene's DNA to form a triple helix. In this method, the presence of the third strand blocks DNA transcription whilst it remains bound.

Chemical modifying groups, for example psoralen cross-linking groups, have been included in TFOs, but these can lead to irreversible DNA damage and mutation. Controlling such chemical modifying groups in cells is also difficult. They may also have disadvantages in relation to cellular delivery of the molecules.

Ribozyme techniques rely on the introduction of a nucleic acid molecule into a cell which expresses a RNA molecule which binds to, and catalyses the selective cleavage of, a target RNA molecule. The target RNA molecule

is typically a mRNA molecule, but it may be, for example, a retroviral RNA molecule.

Antisense- and ribozyme-based techniques have proven difficult to implement and they show varying degrees of success in target gene suppression or inactivation. Furthermore, these two techniques require persistent expression or administration of the gene-inactivating agent.

Linkage of a TFO to a VP16 viral activation domain (Kusnetsova *et al* (1999) *Nucleic Acids Res* **20**, 3995-4000) has been used to broaden the application of TFOs to include gene activation (as opposed to previous uses in gene suppression or inactivation).

Targeted gene deletion by homologous recombination requires two gene-inactivating events (one for each allele) and is not easily applicable to primary cells, particularly for example primary human mammary cells which can only be maintained in culture for a few passages. Targeted gene deletion has remained difficult to perform in plants. The *cre-lox* mediated site-specific integration has been the method of choice although the efficiency of specific integrative events is low (Alberts *et al* (1995) *Plant J.* **7**, 649-659; Vergunst & Hooykass (1998) *Plant Mol. Biol.* **38**, 393-406; Vergunst *et al* (1998) *Nucl. Acids Res.* **26**, 2729-2734).

WO 01/02019 describes methods for inactivating a selected gene using a polypeptide comprising a nucleic acid binding portion which binds to a site present in a eukaryotic genome and a chromatin inactivation portion, for example HDAC or a HDAC recruiter such as PLZF.

The current methods for inducing cell death are unsatisfactory. We consider this to be because they fail to produce an effect on gene expression which is sustained enough or complete enough to kill the cells. If the cell's apoptotic machinery does not work quickly, effectively and for long enough cells usually develop resistance very quickly. These major shortcomings in existing technologis and methods have led us to seek an alternative strategy.

A first aspect of the invention provides a method for promoting (including inducing or restoring) apoptosis in a cell, the method comprising the step of introducing into the cell a molecule comprising (1) a nucleic acid binding portion which binds to a site at or associated with a selected apoptosis-related gene which site is present in a genome and (2) a modifying portion, wherein the nucleic acid binding portion comprises an oligonucleotide or oligonucleotide mimic or analogue, and wherein the modifying portion comprises a polypeptide or peptidomimetic.

In an embodiment the modifying portion is an expression repressor portion. The modifying portion may be capable of modulating covalent modification of nucleic acid or chromatin.

The apoptosis-related gene is a gene involved in controlling apoptosis in a cell, as discussed further below. The apoptosis-related gene may be a gene whose product is able to rescue the cells from apoptosis, including being able to prevent apoptosis occurring (including being able to prevent the completion of apoptosis, even if initiated) in a cell exposed to an apoptotic stimulus.

A second aspect of the invention provides a molecule comprising (1) a nucleic acid binding portion which binds to a site at or associated with a

selected apoptosis-related gene which site is present in a genome and (2) a modifying portion, wherein the nucleic acid binding portion comprises an oligonucleotide or oligonucleotide mimic or analogue and the modifying portion comprises a polypeptide or peptidomimetic. As for the first aspect of the invention, the modifying portion may be an expression repressor portion. The modifying portion may be capable of modulating covalent modification of nucleic acid or chromatin.

It is preferred that the cell or genome is a eukaryotic cell or genome, for example a fungal, animal or plant cell.

The selected gene target (apoptosis-related gene) is involved in controlling cell growth or cell death in any manner. The apoptosis-related gene may be a gene whose overexpression is sufficient on its own (at least under certain conditions) to increase the proportion of cells undergoing apoptosis, when compared with otherwise identical cells in which the gene is not overexpressed (or whose underexpression is sufficient on its own (at least under certain conditions) to decrease the proportion of cells undergoing apoptosis). The gene may be sufficient to promote apoptosis (or its underexpression sufficient to decrease apoptosis) in otherwise normal or unmodified cells. Alternatively, the gene may promote apoptosis (or underexpression decrease apoptosis) only in cells in which a modification has been made to another gene, for example as a result of artificial or natural mutation/selection (for example in a tumour cell) or modification of expression in some other way, for example using a molecule with a DNA binding portion and a modifying molecule as described above.

In such cases, it is desirable to increase the expression of the gene in order to promote apoptosis.

Alternatively, the apoptosis-related gene may be a gene whose overexpression is sufficient on its own (at least under certain conditions) to decrease the proportion of cells undergoing apoptosis, when compared with otherwise identical cells in which the gene is not overexpressed (or whose underexpression is sufficient on its own (at least under certain conditions) to increase the proportion of cells undergoing apoptosis). The gene may be sufficient to decrease apoptosis (or underexpression sufficient to increase apoptosis) in otherwise normal or unmodified cells. Alternatively, the gene may decrease apoptosis (or underexpression sufficient to increase apoptosis) only in cells in which a modification has been made to another gene, for example as a result of artificial or natural mutation/selection (for example in a tumour cell) or modification of expression in some other way, for example using a molecule with a DNA binding portion and a modifying molecule as described above.

In such cases, it is desirable to decrease the expression of the gene in order to promote apoptosis. Such genes (and repression of their expression) are considered to be particularly suitable targets in the methods and molecules of the invention.

It is preferred that the modifying molecule is targeted to a gene whose product is involved in regulation of cell growth or cell death, rather than a gene involved in core cellular activity, whose disruption would be expected to lead to necrotic cell death, as opposed to "programmed" cell death (apoptosis and any other forms of controlled cell death, which may generally be included within the term apoptosis). Examples of suitable target genes are discussed further below. Suitable target genes are mentioned in, for example Sellers & Fisher (1999) *The Journal of Clinical investigation*, p.

1655; Apoptosis and cancer Drug Targeting; and in Cory & Adams (2002) *Nature Reviews Cancer*, p 489; The Bcl-2 family: regulators of the ceullular life-or-death switch.

In embodiments, in the presence of the molecule of the invention the cell viability is 1.2-fold, 1.4-fold, 1.6-fold, two-fold, three-fold, five-fold, ten-fold, twenty-fold, 50-fold, 100-fold, or 1000-fold lower than in the absence of the molecule of the invention under equivalent conditions. The cell viability or cell death can be measured using any suitable technique including cell counting, viability assay or microscopic examination. Apoptosis may be distinguished from necrotic cell death by use of Terminal deoxytransferase-mediated dUTP nick-end labelling (TUNEL). High nick end-labelling reflects the type of DNA fragmentation seen in apoptosis but not in necrotic cell death. Apoptosis may also be detected by measuring an early apoptosis marker on the cell surface, such as Annexin V. This can be done by, for example, staining and FACS sorting.

An inducer of apoptosis (on susceptible cells) may be used as a positive control, and/or an apoptosis inhibitor (on cells susceptible to the inhibitor) may be used as a negative control. For example, staurosporin may be used as an inducer of apoptosis and Zvad or other caspase inhibitors as inhibitors of apoptosis. When the modifying portion is a histone deacetylase or histone deacetylase recruiter (as discussed further below), a deacetylase inhibitor may counter the effect of the molecule of the invention.

It is preferred that the repressor portion or modifying portion is capable of modulating covalent modification of nucleic acid or chromatin.. It is preferred that the repressor or modifying portion is a chromatin inactivation portion. The chromatin inactivation portion may be any polypeptide or part

thereof which directly or indirectly leads to chromatin inactivation. By "directly" leading to chromatin inactivation we mean that the polypeptide or part thereof itself acts on the chromatin to inactivate it. By "indirectly" leading to chromatin inactivation we mean that the polypeptide or part thereof does not itself act on the chromatin but rather it is able to recruit or promote a cellular component to do so.

Chromatin inactivation generally results in the suppression or inactivation of gene expression. Chromatin inactivation is typically a localised event such that suppression or inactivation of gene expression is restricted to, typically, one or a few genes. Thus, the chromatin inactivation portion is any suitable polypeptide which, when part of the molecule of the invention and when targeted to a selected gene by the nucleic acid binding portion, locally inactivates the chromatin associated with the selected gene so that expression of the gene is inactivated or suppressed. Histone deacetylation is associated with chromatin inactivation and so it is particularly preferred if the chromatin inactivation portion facilitates histone deacetylation. Targeted deacetylation of histones associated with a given gene leads to gene inactivation in an, essentially, irreversible manner. By "suppression" or "inactivation" of gene expression we mean that in the presence of the molecule of the invention the expression of the selected, targeted gene is 1.2-fold, 1.4-fold, 1.6-fold, two-fold, three-fold, five-fold, ten-fold, twenty-fold, 50-fold, 100-fold, or 1000-fold lower than in the absence of the molecule of the invention under equivalent conditions. Gene expression can be measured using any suitable method including using reverse transcriptase-polymerase chain reaction (RT-PCR), RNA hybridisation, RNAse protection assays, nuclear run-off assays and alteration of chromatin as judged by DNAse 1 hypersensitivity.

In animal and plant cells histone deacetylation is brought about by the so-called histone deacetylase complex (HDAC) which contains, in addition to one or more histone deacetylase enzymes, ancillary proteins which are involved in the formation and function of the complex. In addition, there are other protein components which although they may not be part of HDAC they bind to or otherwise interact with HDAC and help facilitate histone deacetylation.

Deacetylation and acetylation of histones is a well-known phenomenon which is reviewed in the following: Chen & Li (1998) *Crit. Rev. Eukaryotic Gene Expression* **8**, 169-190; Workman & Kingston (1998) *Ann. Rev. Biochem.* **67**, 545-579; Perlmann & Vennstrom (1995) *Nature* **377**, 387- ; Wolfe (1997) *Nature* **387**, 16-17; Grunstein (1997) *Nature* **389**, 349-352; Pazin & Kadonaga (1997) *Cell* **89**, 325-328; DePinho (1998) *Nature* **391**, 533-536; Bestor (1998) *Nature* **393**, 311-312; and Grunstein (1998) *Cell* **93**, 325-328.

The polypeptide composition of the HDAC complex is currently under investigation. Polypeptides which may form part of, or are associated with, certain HDAC complexes include histone deacetylase 1 (HDAC1) Taunton *et al* (1996) *Nature* **272**, 408-441); histone deacetylase 2 (HDAC2) (Yang *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 12845-12850); histone deacetylase 3 (HDAC3) (Dangond *et al* (1998) *Biochem. Biophys. Res. Comm.* **242**, 648-652); N-CoR (Horlein *et al* (1995) *Nature* **377**, 397-404); SMRT (Chen & Evans (1995) *Nature* **377**, 454-457); SAP30 (Zhang *et al* (1998) *Molecular Cell* **1**, 1021-1031). Sin3 (Ayer *et al* (1995) *Cell* **80**, 767-776; Schreiber-Agus *et al* (1995) *Cell* **80**, 777-786) SAP18 (Zhang *et al* (1997) *Cell* **89**, 357-364); and RbAp48 (Qian *et al* (1993) *Nature* **364**, 648-652). All of these papers are incorporated herein by reference. It is believed that there

may be further components of the HDAC complex or which interact with the HDAC complex which are, as yet, undiscovered. It is envisaged that these too will be useful in the practice of the invention.

PLZF has been shown to interact with N-CoR and SMRT, which in turn recruit a HDAC complex. PLZF will also directly interact with HDAC (Lin *et al* (1998) *Nature* **391**, 811-814; Grignani *et al* (1998) *Nature* **391**, 815-818; David *et al* (1998) *Oncogene* **16**, 2549-2556).

Mad1 is a member of the Mad family and has an ability to act as a transcriptional repressor. It has been shown that Mad1 is able to interact with Sin3, which in turn interacts with class I histone deacetylases (HDAC1 and HDAC2). Mad/Sin3 functions as a large protein scaffold capable of multiple protein – protein interactions (Hassig et al (1997) Cell 89, 341-347; Laherty et al (1997) Cell 89, 349-356; Zhang et al (1997) Cell 89, 357-364)).

Complexes formed which contain any of N-CoR, SMRT, Sin3, SAP18, SAP30 and histone deacetylase are described in Heinzel *et al* (1997) *Nature* **387**, 43-48; Alland *et al* (1997) *Nature* **387**, 49-55; Hassig *et al* (1997) *Cell* **89**, 341-347; Laherty *et al* (1997) *Cell* **89**, 349-356; Zhang *et al* (1997) *Cell* **89**, 357-364; Kadosh & Struhl (1997) *Cell* **89**, 365-371; Nagy *et al* (1997) *Cell* **89**, 373-380; and Laherty *et al* (1998) *Molecular Cell* **2**, 33-42. All of these papers are incorporated herein by reference.

Thus, it is particularly preferred if the component of a HDAC complex or the polypeptide which binds to or facilitates recruitment of a HDAC complex is any one of MAD1, E7, PLZF, SMRT, Sin3, SAP18, SAP30 or N-CoR, or HDACs including HDAC1, HDAC2 or HDAC3, or NuRD,

MAD2, MAD3, MAD4 or Rb. It will be appreciated that it may not be necessary for all of the polypeptides to be present so long as a functional portion thereof is present. For example, with respect to histone deacetylase enzymes (for example, HDAC1, HDAC2 or HDAC3) the functional portion may be a portion that retains histone deacetylase activity or it may be a portion which contains a binding site for other components of a HDAC complex or a portion which otherwise recruits the HDAC complex and promotes histone deacetylation. Similarly, with respect to other components of the HDAC complex or polypeptides which bind to the HDAC complex the functional portion may be a portion which contains a binding site for other components of the HDAC complex. To date six mammalian HDAC genes have been described (Grozinger *et al* (1999) *Proc. Natl. Acad. Sci. USA* 96, 4868-4873), it is believed that any one or more of these may be useful in the practise of the present invention.

The modifying portion may be VP16 or KRAB, though this is not preferred. Thus, in an embodiment, VP16 or KRAB are not included within the meaning of the term "modifying portion" or "chromatin inactivation portion". VP16 is a transcriptional activator whose mode of action is not considered to involve covalent modification of DNA or chromatin. KRAB is a transcriptional repressor whose mode of action is considered to involve mechanisms other than chromatin inactivation. Although not preferred, any fragment of KRAB that, when part of the molecule/polypeptide as defined above and when targeted to a selected gene by the nucleic acid binding portion, locally inactivates the chromatin associated with the selected gene so that expression of the gene is inactivated or suppressed, is included within the term "chromatin inactivation portion". For example, any fragment of KRAB that is capable of binding to or facilitating recruitment of a HDAC

complex is included within the term "chromatin inactivation portion". However, any such fragments are not preferred.

It is believed that binding motifs are present within the components of the HDAC complex or within polypeptides which bind the HDAC complex and these motifs may be sufficient to act as chromatin inactivation portions in the polypeptide of the invention since they may facilitate histone deacetylation by recruiting a HDAC complex.

Furthermore, it will be appreciated that variants of a component of the HDAC complex or variants of a polypeptide which binds to the HDAC complex may be used. Suitable variants include not only functional portions as described above, but also variants in which amino acid residues have been deleted or replaced or inserted provided that the variant is still able to facilitate histone deacetylation. Thus, suitable variants include variants of histone deacetylase in which the amino acid sequence has been modified compared to wild-type but which retain their ability to deacetylate histones. Similarly, suitable variants include variants of, for example, Sin3 or PLZF in which the amino acid sequence has been modified compared to wild-type but which retain their ability to interact with or in the HDAC complex. Similarly, variants of other proteins interacting with components of the HDAC complex and other transcription factors that can bring about gene inactivation through HDAC activity may be used.

All or parts of the Rb, MAD and MeCpG2 proteins may interact with the HDAC complex.

While most work has been done on HDAC complexes and polypeptides involved in recruiting HDAC complexes in mammalian systems, the

fundamental nature of the system is such that functionally equivalent polypeptides are expected to be found in other eukaryotic cells, in particular in other animal cells and in plant cells. For example, Figure 5 shows that polypeptides very closely related to human HDAC1 are present in arabidopsis and in yeast. A plant HDAC complex has been isolated from maize (Lussen *et al* (1997) *Science* **277**, 88-91) and a comparative study of histone deacetylases from plant, fungal and vertebrate cells has been undertaken (Lechner *et al* (1996) *Biochim. Biophys. Acta* **1296**, 181-188). Histone deacetylase inhibitors have been shown to derepress silent rRNA genes in Brassica (Chen & Pickard (1997) *Genes Dev.* **11**, 2124-2136) and a naturally occurring host selective toxin (HC toxin) from *Cochliobolus carbonum* inhibits plant, fungal and mammalian histone deacetylases (Brosch *et al* (1995) *Plant Cell* 7, 1941-1950).

It is not necessary that the chromatin inactivation portion is from the same cell type or species as the cell into which the molecule is introduced but it is desirable if it is since such a chromatin inactivation portion may be able to inactivate chromatin more effectively in that cell.

It is particularly preferred if the chromatin inactivation portion of the molecule is PLZF, E7, MAD1, Rb or SAP18, or a portion of PLZF or E7 or MAD1 or Rb or SAP18 that can facilitate histone deacetylation, or a polypeptide, or portion of a polypeptide, known to cause gene activation *via* histone deacetylation. For example, the portion of PLZF in PLZF-RAR_ which is involved in APL is believed to interact with N-CoR and SMRT.

Preferred chromatin inactivation portions are described in the Examples, and include a polypeptide/polypeptide mimic or analogue derivable from SAP18 with the amino acid sequence

XXXMAVESRVTQEEIKKEPEKPIDREKTCPLLLRVF (where XXX is, for example, a AAA or DDD linker, or other hydrophilic, preferably charged linker) and a polypeptide derivable from MAD1 with the amino acid sequence XXXMNIQMLLEAADYLERREREAEHGYASMLP (where XXX is, for example, a AAA or DDD linker, or other hydrophilic, preferably charged linker).

It is also particularly preferred if the chromatin inactivation portion is a polypeptide with histone deacetylase enzyme activity such as contained in HDAC1, HDAC2 or HDAC3.

Alternatively, the modifying portion may be a portion that is capable of modulating covalent modification, for example methylation, of nucleic acid, preferably DNA. Thus, the modifying portion may be or comprise a DNA modifying enzyme, or may be capable of recruiting such an enzyme. The modulation preferably has the effect of suppressing the selected gene.

It is preferred that the modifying portion does not change the sequence of the nucleic acid. It is preferred that the modifying portion does not cleave the nucleic acid backbone. The modifying portion is preferably not a recombinase or a restriction endonuclease.

For example, the modifying portion may comprise (or be capable of recruiting) all or a portion of a methyl transferase or a component of a methyltransferase complex, for example as discussed in Okano M, Xie S, Li E. (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 19:219-220; Adrian P. Bird and Alan P. Wolffe (1999) Methylation-Induced Repression: Belts, Braces, and Chromatin. *Cell* 99, 451-454.

It is preferred that the repressor or modifying portion is not an endonuclease or other molecule that produces a persistent break in the DNA strand.

It is preferred that a polypeptide/polypeptide mimic or analogue portion of the molecule (for example the modifying portion) has a molecular mass of less than 11 kDa, preferably less than 8 kDa, still more preferably less than 6 kDa. For example, it is preferred that the polypeptide/polypeptide mimic or analogue portion has less than 100, still more preferably less than 90, 80, 70, 60, 50, 45, 40, 35, 30, 25 or 20 amino acids (or mimics or analogues thereof), most preferably between about 60 and 25 amino acids (or mimics or analogues thereof).

It is particularly preferred that the modifying portion consists of peptides derivable from SAP18 or MAD1 or Rb and appropriate linkers, for example the peptides derivable from SAP18 or MAD1 and linkers as described above and in Example 1.

The molecule may further comprise a portion which facilitates cellular entry and/or nuclear localisation (locating portion). This portion may also be a polypeptide or polypeptide mimic/analogue. For example, the locating portion may comprise or consist of a peptide with membranotropic activity as discussed, for example, in Soukchareun *et al* (1998) *Bioconjugate Chem* **9**, 466-475 and references cited therein, for example Soukchareun *et al* (1995) *Bioconjugate Chem* **6**, 43-53 (viral fusion peptides) or Eritja *et al* (1991) *Tetrahedron* **47**, 4113-4120 (nuclear transport signal sequences). It may be a nuclear localisation signal peptide (for example DDDPKKKRKV-NH_2) or endosomal lytic peptide (which may facilitate release of the molecule from the endosomal compartment) mentioned in WO 99/13719. It

is preferred that this portion is of less than 3 kDa, preferably of less than 2.5 kDa. It is preferred that the total polypeptide/mimic/analogue content of the molecule is less than 11 kDa. Typically, a localisation portion may have between about 7 and 16 amino acids.

Further examples of localisation portions include modified Antennapedia homeodomain based Penetratins (for example RQIKIWFQNRRMKWKK), or TAT (for example C(Acm)GRKKRRQRRRPPQC, where C(Acm) is a Cys-acetamidomethyl) or VP22 based molecules (Prochiantz (2000) *Curr Opin Cell Biol* 9, 420-429).) or basic HIV TAT internalisation peptide.

The molecules of the invention may be useful in methods and uses provided by aspects of the invention, for example as discussed in more detail below. In particular, the polypeptides of the invention may be useful in a method of the first aspect of the invention.

It is preferred if the molecules of the invention are hybrid molecules which do not occur in nature. For example, it is preferred if the nucleic acid binding portion and the modifying portion are not derivable from a naturally occurring complex or molecule. The molecules (if any) from which the nucleic acid binding portion and the chromatin inactivation portion are derived may be from the same species (for example, as is described in more detail below, the nucleic acid binding portion may be an oligonucleotide having a sequence found in human nucleic acid and the chromatin inactivation portion may be a portion of human PLZF) or they may be from different species (for example an oligonucleotide having a sequence not found in human nucleic acid, for example capable of binding to a bacterial DNA sequence, may be fused to a portion of human PLZF).

Thus, in a particular preferred embodiment the molecule of the invention is one which is produced by chemical synthesis methods wherein the nucleic acid binding portion and the modifying or chromatin inactivation portion are selected as is described in more detail below.

Synthesis and joining techniques are discussed in WO 01/14737 and references therein (incorporated herein by reference). The methods of WO 01/147373 are preferred. Alternatively, techniques described in Kusnetsova *et al* (1999) *Nucleic Acids Res* **27**, 3995-4000 may also be used.

The site present in a eukaryotic genome is a site which is at or associated with a selected gene or genes whose expression it is desirable to modulate, preferably suppress or inactivate. It is preferred if the site is a site which is naturally present in a eukaryotic genome. However, as is discussed in more detail below, the site may be one which has been engineered into the genome, or it may be a site associated with an inserted viral sequence. The site engineered into the genome to be in the vicinity of the gene whose expression is to be suppressed may be a site from the same species (but present elsewhere in the genome) or it may be a site present in a different species. By "genome" we include not only chromosomal DNA but other DNA present in the eukaryotic cell, such as DNA which has been introduced into the cell, for example plasmid or viral DNA. It is preferred if the nucleic acid binding portion can bind to chromosomal DNA or, as is described in more detail below, to RNA transcribed from chromosomal DNA.

In an embodiment, it is preferred that the gene is an endogenous gene. The term "endogenous gene" refers to a gene that is native to the cell ie which is not heterologous to the cell and is in its natural genomic context. In this context the site present in a eukaryotic genome is a site which is at or

associated with the selected endogenous gene or genes whose expression it is desirable to suppress or inactivate. The site is a site which is naturally present in a eukaryotic genome and is in its natural genomic context.

As noted above, the gene (including its product) is associated with cell death or viability control. This may be either directly or indirectly and the effect may take place at any time after treatment with the agent.

It may be desirable for the site to have particular sequence characteristics that promote binding to an oligonucleotide to form a triple helix, as known to those skilled in the art. However, it is considered in relation to the present invention that such sequence characteristics may be less important than for oligonucleotides in the absence of a polypeptide portion, because the suppressing or modulating effect of the molecule of the invention may persist even when the molecule is no longer bound to the target site; thus the affinity of binding may be less critical. The sequence of the oligonucleotide is still important so that specific recognition is obtained; however the bonds that are formed between oligo and target sequence may not need to be as strong when the polypeptide/peptidomimetic portion is present.

Positioning of the oligonucleotide binding site relative to the gene whose transcription is to be suppressed or modulated may also be less critical than for oligonucleotides, for example TFOs, without a modifying portion as the modulating or suppressing effect of the molecule of the invention (for example when the modifying domain is or is capable of recruiting a methyltransferase or histone deacetylase) may extend to either side of the oligonucleotide binding site. The nucleic acid binding portion may bind to the gene promoter, but may alternatively bind to another sequence within or in proximity to the gene of interest.

WO 90/06934/ EP 0 375 408 and WO 91/06626 discuss sequence requirements for TFOs. Two motifs for the formation of a triple helix are termed the "CT" motif and the "GT" motif. The first of these involves the use of a polypyrimidine oligonucleotide as the TFO. For every GC base pair, a C is present in the TFO and for every AT base pair, a T (or xanthine or inosine or a halogenated derivative) is present in the TFO. The TFO is considered to be oriented in a parallel direction to the purine-rich strand of the duplex. Alternatively, using the "GT" motif, a G (or halogenated derivative) is present for every GC base pair and a T (or xanthine or inosine or a halogenated derivative) for each AT base pair, and the TFO is considered to be oriented in an anti-parallel direction to the purine-rich strand of the duplex. The target sequence should have at least about 65% purine bases or at least about 65% pyrimidine bases. EP 0 266 099 also discusses how suitable target sequences may be selected.

WO94/17086 discusses oligonucleotides that are intended to bind to DNA sequences that are considered to be capable of adopting a single-stranded conformation. Such sequences may be purine-rich and have substantial mirror symmetry. The oligonucleotides may be substantially complementary to the purine strand, or may have a circular or stem-loop functioning structure that may form both Watson-Crick and Hoogensteen bonds with the single-stranded target DNA.

WO96/35706 describes oligonucleotides with structures and sequence characteristics that are considered to promote specific and stable complex formation with target nucleic acid (pyrimidine single-stranded nucleic acids) and which may have greater stability due to formation of a parallel-stranded hairpin structure in the absence of target nucleic acid.

Debin *et al* (1999) *Nucl Acids Res* **27(13)**, 2699-2707 comments on factors affecting the stability of G,A triple helices and the consequences for TFO design. Xodo *et al* (2001) *Eur J Biochem* **268**, 656-664 also investigates factors affecting TFO binding to target sites, for example binding of short oligonucleotides to neighbouring sites.

Blume *et al* (1999) *Nucl Acids Res* **27**, 695-702 investigates the involvement of a divalent cation in triple helix formation and how formation may be positively or negatively modulated. Faria *et al* (2001) *J Mol Biol* **306**, 15-24 describes an assay for evaluating TFOs in cells and results with various oligonucleotides. Cheng *et al* (2000) *Biotech and Bioeng* **70**, 467-472 presents the results of mathematical modelling of TFO bindings and the consequences for choosing binding sites and TFO sequences.

Demidov & Frank-Kamenetskii review binding of peptide nucleic acids (PNAs), particularly cationic pyrimidine PNAs (cpyPNAs) to duplex DNA.

Rules for designing potential TFOs are reviewed in Vasquez & Wilson (1998) *Trends Biochem Sci* **1**, 4-9. Three types of TFOs are indicated to be effective: pyrimidine rich (CT); purine rich (GA) and mixed (GT or GAT). CT TFOs bind in a parallel motif, in which the third strand has the same 5' to 3' orientation as the purine strand of the duplex. GA TFOs bind in an antiparallel motif. Mixed TFOs may bind in either manner, depending on the target sequence. Other properties also differ between the types of TFOs; for examkple CT TFOs are pH dependent. Each type of TFO may be suitable in relation to the present invention.

It is preferred that the oligonucleotide or mimic portion is about 10 to 80, preferably 15 to 40 bases long, still more preferably about 20 to 40 bases long. Oligonucleotides of less than 20 bases may display weaker and/or less specific binding but may nevertheless be useful in the practice of the invention, for example because only transient binding is required, as noted above.

By "DNA" or "oligo(deoxy)nucleotide" we mean a molecule with a sugar-linkage-sugar backbone wherein the sugar residue comprises a 2'-deoxyribose (and therefore includes a DNA chain terminated with a nucleoside comprising a 2',3' dideoxyribose moiety) and wherein, attached to the sugar residue at the 1 position is a base such as adenine (A), cytosine (C), guanine (G), thymidine (T), inosine (I), uridine (U) and the like. In normal DNA the linkage between sugar residues (the "sugar-sugar linkage") is a phosphate moiety which forms a diester with the said sugar residues. However, we include in the term "nucleic acid" (and more particularly in the term DNA) molecules with non-phosphate linkages.

Thus, we include a phosphorothioate linkage and a phosphoroselenoate linkage. It may be preferred that the linkages are more resistant to attack by cellular nucleases than normal DNA. Such linkages may also include methyl phosphate, phosphotriester and the _ enantiomer of naturally occurring phosphodiester.

By the terms "nucleic acid" or "oligonucleotide" we also include molecules with non-natural base analogues; molecules in which the 2' and 3' positions of the pentose sugar are independently any of -H, -OH or $-NH_2$; and molecules in which an oxygen attached to the phosphorus atom but not in

phosphodiester linkage is replaced by -SH, SeH, $-BH_2$, $-NH_2$, $-PH_3$, -F, -Cl, $-CH_3$, $-OCH_3$, -CN and -H.

The oligonucleotide may be a oligoribonucleotide or a oligodeoxyribonucleotide. Oligodeoxyribonucleotides are preferred as oligoribonucleotides may be more susceptible to enyzymatic attack than oligodeoxyribonucleotides.

The oligonucleotide or analogue or mimic may be a peptide nucleic acid, as known to those skilled in the art and described in, for example WO 99/13719 and references therein, and in Demidov & Frank-Kamenetskii (2001) *supra*. PNAs are nucleic acid analogs with a polyamide (peptide) backbone containing 2-aminoethyl glycine units in place of the deoxyribose-phosphate backbone of DNA. The PNA backbone is neutral (unlike the DNA backbone, which is negatively charged) and may therefore bind more stably to a charged nucleic acid molecule than would the corresponding DNA molecule.

It is preferred that the oligonucleotide or analogue or mimic is a DNA oligonucleotide.

References to an oligonucleotide include (where appropriate) reference to an oligonucleotide mimic or analogue, for example a PNA.

The oligonucleotide may comprise a linker, which may be attached to the 5' or 3' terminus of the oligonucleotide. Examples of suitable linkers are described in, for example, WO 90/06934.

It may be preferred that the nucleic acid binding portion is or comprises a peptide nucleic acid (PNA).

The nucleic acid binding portion may be any suitable binding portion as defined which binds to a site present in a eukaryote, such as a plant or animal, genome. It is particularly preferred that the nucleic acid binding portion is able to bind to a site which is at or associated with a selected gene whose expression is to be modified, particularly suppressed by the presence of the chromatin inactivating portion of the molecule of the invention. It is preferred that the nucleic acid binding portion binds selectively to the desired site. There may be one or more desired sites to which the nucleic acid binding portion may bind. Typically there is one intended target site in the target genome. For the avoidance of doubt, the site present in the eukaryote may most usefully be a naturally occurring site, or it may be a site which has been engineered to be there. The site need not be originally from the same or any other eukaryote. For example, it may be a bacterial or viral sequence or artificial sequence for which TFOs have previously been characterised, which has been engineered to be present in the DNA of the eukaryotic cell, for example a plant cell. Examples may include response elements, such as ERE and IRE as described in examples here, or other characterised binding sites. It may be desirable to use such a site in a cell which does not contain an endogenous regulator of the site. Alternatively, the site may be a modified version of a naturally occuring response element, which modified version may serve as a binding sites for TFOs, but may not be regulated by a naturally occuring regulator of the naturally occuring response element. However, it is preferred if the site to which the nucleic acid binding portion binds is naturally present in the eukaryotic cell and is present in its natural position in the genome.

The nucleic acid binding portion may be a DNA binding portion or an RNA binding portion. Thus, the nucleic acid binding portion may bind to double-stranded nucleic acid (for example DNA) or to single-stranded nucleic acid (for example RNA or single-stranded DNA). In the case of the RNA binding portion, the site present in the eukaryotic genome which binds the RNA binding portion is, typically, nascent RNA being transcribed from DNA at the selected site for inactivation. The RNA may be that which is being transcribed by the gene whose expression is to be suppressed, or it may be that which is being transcribed by a gene adjacent to, or at least close to, the gene whose expression is to be suppressed. It is preferred that the RNA binding portion binds to an RNA sequence which is at or close to the 5' end of the transcript. It will be appreciated that whilst being transcribed, nascent RNA remains at or close to its site of transcription and that if the site of transcription is at or close to the gene whose expression is to be suppressed, using an RNA binding portion in the molecule of the invention facilitates the localisation of the chromatin inactivation portion to the desired site.

It may be useful if the DNA binding portion binds to a transcription factor binding site, for example so that expression of more than one gene to which the transcription factor binds may be modulated. Transcription factors associated with apoptotic genes include CREB, WT1, NF-kappaB and Stat3. Databases listing transcription factors and their binding sites are listed below:

http://www.embl-heidelberg.de/srs5bin/cgi-bin/wgetz?-fun+pagelibinfo+-info+TFFACTOR
http://www.embl-heidelberg.de/srs5bin/cgi-bin/wgetz?-fun+pagelibinfo+-info+TFSITE

http://www.embl-heidelberg.de/srs5bin/cgi-bin/wgetz?-fun+pagelibinfo+-info+TFCELL

http://www.embl-heidelberg.de/srs5bin/cgi-bin/wgetz?-fun+pagelibinof+-info+TFCLASS

http://www.embl-heidelberg.de/srs5bin/cgi-bin/wgetz?-fun+pagelibinfo+-info+TFMATRIX

http://www.embl-heidelberg.de/srs5bin/cgi-bin/wgetz?-fun+pagelibinfo+-info+TFGENE

It may be useful if the DNA binding portion binds to a promoter region or other regulatory regions or sequences just upstream of the transcription start site. In some applications it may be preferred to target sequences within the gene in order to differentiate amongst splice variants.

As noted, oligonucleotides may be designed/engineered so as to bind to a particular, selected target DNA sequence which is at or associated with a selected gene. In one embodiment of the invention the oligonucleotide is one which has been engineered to bind to a site which is present in a mutant gene sequence within the plant or animal cell but is not present in the equivalent wild type sequence. For example, and as is discussed in more detail below, the oligonucleotide may bind selectively to a dominant negative, mutated gene, such as a mutant apoptosis-related oncogene and, upon binding, DNA methylation or chromatin inactivation occurs and suppresses the expression of the mutant apoptosis-related oncogene. Examples of apoptosis-related genes that are mutated include Protein kinase B/AKT and PTEN (Hill M and Hemmings B (2002): Inhibition of protein kinase B/AKT. Implications for cancer therapy. *Pharmacol Ther*, 93, p. 243; Kanaseki T *et al* (2002) Identification of germline mutation of PTEN gene

and analysis of apoptosis resistance of the lymphocytes in a patient with Cowden Disease. *Pathobiology* **70**, p. 34).

RAS (*H-ras*) and *Bcl-10* are excluded from the definition of apoptosis-related genes.

Typically, the nucleic acid binding portion and the modifying or chromatin inactivation portion are fused. The nucleic acid binding portion and modifying or chromatin inactivation portion may be synthesised as a single molecule (total synthesis approach), for example by consecutive assembly of the peptide and then the oligonucleotide on a solid support, for example as described in Soukchareun *et al* (1998) *supra* and references cited therein, or in Basu *et al* (1995) *Tetrahedron Lett* **36**, 4943. Preferably, an automated procedure is used.

Alternatively, the nucleic acid binding portion and modifying or chromatin inactivation portion are synthesised separately, using techniques well known to those skilled in the art, and then joined. Techniques suitable for the coupling of peptide nucleic acids to peptides include the use of heterobifunctional conjugation reagents such as SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate) and SMCC (succinimidyl 4-(N-maleimidomethyl) cylohexene-carboxylate) and are described, for example, in WO 99/13719, particularly in Examples 12 to 15. Techniques suitable for coupling oligodeoxynucleotides to peptides include the use of N_-Fmoc-cysteine(*S*-thiobutyl) derivatised oligodeoxynucleotides, as described in Soukchareun *et al* (1998) *supra*. Other techniques include the use of *N*-hydroxybenzotriazole (HOBT) ester activation of the 3' or 5' ends of oligonucleotide phosphates prior to coupling of an unprotected peptide *via* a nucleophilic group (such as an _-NH_2 group) in the peptide (see

Ivanovskaya *et al* (1995) *Nucl Nucl* **6**, 931-934; Ivanovskaya *et al* (1987) *Dokl Acad Nauk SSSR* **293**, 477-481; Kuznetsova *et al* (1999) *Nuc Acids Res* **27**, 3995-4000). Peptide-oligonucleotide conjugation techniques are reviewed in, for example, Tung & Stein (2000) *Bioconjugate Chem* **11(5)**, 605-618.

Preferably, a "native ligation" technique is used, as described in WO 01/15737 and Stetsenko & Gait (2000) *Organic Chem* **65(16)**, 4900-4908. A N-terminal thioester-functionalised peptide is ligated to a 5'-cysteinyl oligonucleotide derivative.

Suitably, the nucleic acid binding portion and the repressor, modifying or chromatin inactivation portion are joined so that both portions retain their respective activities such that, for example, the nucleic acid binding portion may bind to a site present in a plant or animal genome and, upon binding, the modifying portion is still able to modulate covalent modification of nucleic acid or chromatin, for example a chromatin inactivation portion is still able to inactivate chromatin. The two portions may be joined directly, but they may be joined by a linker peptide or oligonucleotide. Suitable linker peptides are those that typically adopt a random coil conformation, for example the polypeptide may contain alanine or proline or a mixture of alanine plus proline residues. Preferably the amino acids promote solubility; thus, the linker may contain, for example, charged or hydrophilic amino acids such as aspartic acid residues. Preferably the linker may contain or consist of aspartic acid residues. It is preferred that the amino acids are not hydrophobic amino acids, such as phenylalanine or tryptophan. Preferably, the linker contains between 10 and 100 amino acid residues, more preferably between 10 and 50 and still more preferably between 10 and 20. A shorter linker, for example of between 3 and 9 amino acids, may also be useful. In

any event, whether or not there is a linker between the portions of the molecule the molecule is able to bind its target nucleic acid and is able to repress expression or modulate covalent modification of nucleic acid or chromatin, for example inactivate chromatin thereby selectively suppressing or inactivating gene expression.

Polynucleotides which encode suitable repressor, modifying or chromatin inactivation portions are known in the art or can readily be designed from known sequences and made. Polynucleotide sequences encoding various suitable chromatin inactivation portions are given above in the references which refer to the polypeptides or are available from GenBank or EMBL or dbEST. A reference for PLZF is Chen *et al* (1993) *EMBO J.* **12**, 1161-1167. A reference for E7 is Tommasino *et al* (1995) *Bioessays* **17**, 509-518. References for SAP18, MAD1 and Rb are respectively Zhang *et al* (1997) *Cell* **89**, 357-364; Ayer *et al* (1993) *Cell* **72**, 211-222 and Weinberg (1995) *Cell* **81**, 323-330.

Polynucleotides which encode suitable linker peptides can readily be designed from linker peptide sequences and made.

Thus, polynucleotides which encode the repressor or modifying portions of the molecules of the invention can readily be constructed using well known genetic engineering techniques. The repressor or modifying portions may therefore be synthesised by expression, using techniques of molecular biology well known to those skilled in the art. However, it may be preferred to synthesise the polypeptide/analogue/mimic portion(s) of the molecules of the invention by techniques of organic chemistry, as known to those skilled in the art and discussed herein.

The present invention also relates to a host cell transformed with a molecule of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include plant, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic and kidney cell lines. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650; 293 cells which are human embryonic kidney cells, and HT1080 human fibrosarcoma cells.

Protoplasts for transformation are typically generated as required by methods known in the art. Plant cell lines are not generally available. However, one cell line which is commonly used is the Bright Yellow 2 cell line from tobacco (BY2; Mu *et al* (1997) *Plant Mol. Biol.* **34**, 357-362).

Transformation of appropriate cell hosts with a molecule of the present invention is accomplished by well known methods that typically depend on the type of molecule used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods*

In Yeast Genetics, A Laboratory Manual, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA. With regard to plant cells and whole plants the following plant transformation approaches (J. Draper and R. Scott in D. Grierson (ed.), "Plant Genetic Engineering", Blackie, Glasgow and London, 1991, vol. 1, pp 38-81) may be used:

i) DNA-mediated gene transfer, by polyethylene glycol-stimulated DNA uptake into protoplasts, by electroporation, or by microinjection of protoplasts or plant cells (J. Draper, R. Scott, A. Kumar and G. Dury, *ibid.*, pp 161-198). Direct gene transfer into protoplasts is also described in Neuhaus & Spangenberg (1990) *Physiol. Plant* **79**, 213-217; Gad *et al* (1990) *Physiol. Plant* **79**, 177-183; and Mathur & Koncz (1998) *Method Mol. Biol.* **82**, 267-276;

ii) transformation using particle bombardment (D. McCabe and P. Christou, *Plant Cell Tiss. Org. Cult.*, **3**, 227-236 (1993); P. Christou, *Plant J.*, **3**, 275-281 (1992)).

Preferred techniques include electroporation, microinjection and liposome formulation.

Some species are amenable to direct transformation, avoiding a requirement for tissue or cell culture (Bechtold *et al* (1993) *Life Sciences*, C.R. Acad. Sci. Paris **316**, 1194-1199).

In all approaches a suitable selection marker, such as kanamycin- or herbicide-resistance, is preferred or alternatively a screenable marker ("reporter") gene, such as β-glucuronidase or luciferase (see J. Draper and R. Scott in D. Grierson (ed.), "Plant Genetic Engineering", Blackie, Glasgow and London, 1991, vol. 1 pp 38-81).

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cell, bacterial cells, insect cells, vertebrate cells and some plant cells (eg barley cells, see Lazzeri (1995) *Methods Mol. Biol.* **49**, 95-106).

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* **2**, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a molecule of the present invention, can be identified by well known techniques. For example, labelled oligos and/or GFP markers may be used.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

It will be appreciated that the transformed cells or culture may be cells or culture in which the molecule of the invention does not promote apoptosis, either because of the nature of the cells (for example because they are recombinant cells in which, for example, there is a recombinant copy of the target gene which differs from the target gene in such a way that the molecule of the invention does not bind the recombinant copy) or because the conditions under which the cells/culture are grown or kept are not such that apoptosis is induced in the cells.

In relation to plants, it is envisaged that the invention includes single cell derived cell suspension cultures, isolated protoplasts or stable transformed plants.

Although the molecules of the invention may be introduced into any suitable host cell, it will be appreciated that they are primarily designed to be effective in appropriate animal or plant cells, particularly those that have one or more sites within their DNA to which the molecule of the invention may bind.

Thus, the animal or plant cells which contain a molecule of the invention whose presence suppresses the expression of a particular gene, or the animals or plants containing these cells, may be considered to have the gene "knocked out" in the sense that it can no longer be expressed. The chromatin inactivation by histone deacetylation may be essentially irreversible without further intervention. Repression by histone deacetylation may be reversed by using an inhibitor of histone deacetylase, for example Trichostatin A (TSA), Trapoxin or sodium butyrate (NaB), as known to those skilled in the art. Similarly, methylation may be essentially irreversible without further intervention, for example administration of

methylation inhibitors/reversers, which are known in the art and include the compound azacytidine. Other methylation inhibitors include 5 deoxy-azacytidine, or, for example, antisense oligos (or gene expression suppressors as described herein) directed to a DNA methyltransferase.

It will be readily appreciated that introduction of a molecule of the invention into an animal or plant cell will allow targeting of the molecule to an appropriate binding site within the nucleic acid, for example DNA (and which is bound by the DNA-binding portion of the polypeptide) and allow for suppression or inactivation or other modulation of gene expression, for example by allowing the chromatin at or associated with the target binding site to be inactivated. Typically, the molecule of the invention is selected so that it targets a selected gene. Thus, suitably, the targeted gene has a site which is bound by the DNA binding portion of the molecule associated with it. The site which is so bound may be within the gene itself, for example within an intron or within an exon of the gene; or it may be in a region 5' of the transcribed portion of the gene, for example within or adjacent to a promoter or enhancer region; or it may be in a region 3' of the transcribed portion of the gene.

The ability to modulate, particular suppress the expression of a selected apoptosis-related gene is useful in many areas of biology.

Typically, when the gene whose expression is suppressed is in an animal cell, the animal cell is a cell within an animal and the method of the invention is used to modulate, for example suppress the expression of a selected apoptosis-related gene in an animal (which may be a human or a non-human animal), thereby modulating, for example promoting, apoptosis. Examples of particular uses in animal cells include inactivation of Bcl-2,

Bcl-XI (a Bcl-2 family member) or AKT/PKB (protein kinase B; various alleles). These genes are involved in control of apoptosis, as described, for example, in Vivanco & Sawyers (2002) The phosphatidylinositol 3-kinase – AKT pathway in human cancer. *Nature Reviews Cancer*, p 489; Cory & Adams (2002) The Bcl-2 family: regulators of the ceullular life-or-death switch.

Accession numbers for the Bcl2, AKT and BCl-XI genes include the following: Bcl2: NM_000657 and NM_000633, AKT: NM_005163, Bcl-XL: NM_138578.

Also typically, the plant cell is a cell within a plant and the method of the invention is used to suppress the expression of an apoptosis-related selected gene in a plant. This may be useful in, for example, in defence mechanisms against invading pathogens (Mittler R. *et al* (1996) Inhibition of Programmed Cell Death in Tobacco Plants during a Pathogen-Induced Hypersensitive Response at Low Oxygen Pressure. *Plant Cell*, 8, p.1991).

Suitably, the method of the invention is used to modulate, suppress or inactivate the expression of an apoptosis-related gene whose expression it is desirable to modulate, suppress or inactivate. Genes whose expression it is desirable to suppress or inactivate include apoptosis rescue genes, for example AKT/PKB, Bcl-XI or Bcl-2 or viral genes including genes present in proviral genomes and so the method in relation to animals may constitute a method of medical treatment. In addition, oncogenes may be overexpressed in certain cancers and it may be desirable to suppress their expression in combination with a further apoptosis-promoting modulation. Some oncogenes are oncogenic by virtue of having an activating mutation.

The selective suppression of expression of a mutant oncogene may be achieved using a DNA binding portion that selectively binds to the mutant oncogene sequence and wherein the repressor or modifying portion, for example chromatin inactivation portion suppresses expression of the mutant oncogene, for example by inactivating the chromatin in which the oncogene resides or with which it is associated. Suppression of oncogene overexpression or of mutant (especially activated) oncogene expression is generally desirable in treating cancers in which the oncogenes play a role. Mutant oncogenes which may be targeted include *Ras* and *Bcl-10*. These may be targeted by DNA binding portions capable of recognising the mutated genes in a sequence specific manner.

Apoptosis is a programmed cell death, which can be induced by various stimuli. For example many chemotherapeutic drugs eliminate cancer cells by induction of apoptosis. Tumor development and progression is thought to require both increased proliferation and inhibition of apoptosis. Primary or acquired resistance to current treatment protocols remains a major concern in clinical oncology and may be caused by defects in apoptosis programs and it is known that resistance to chemotherapy is partly due to a decreased apoptosis rate. Several genes have been reported to be involved with control of apoptosis and for example expression of the bcl-2 proto-oncogene is found in various human hematologic malignancies and solid tumors. Bcl-2 protein exerts its oncogenic role by preventing tumor cells from undergoing apoptosis induced by radiation, chemotherapy, and hormonal therapy. Therefore it is desirable to develop therapies, which aim to prevent the expression of the genes, which are involved in prevention of apoptosis.

These methods of the invention typically involve the transfer of the molecule of the invention into an animal or plant cell.

Tranfer systems useful with oligonucleotides or oligonucleotide-peptide fusions will be known to those skilled in the art and may be useful in the practice of the methods of the present invention in which the molecule of the invention is introduced into a cell either within or outwith an animal body.. For example, liposome or virus-based methods may be used. Electroporation (see, for example, Kuznetsova *et al* (1999) *Nucl Acids Res* **27(20)**, 3995-4000), ballistic methods, cationic lipids (for example as described in Felgner *et al* (1997) *Hum Gene Ther* **8**, 511-512 or WO 99/13719) or specific ligands attached to the oligonucleotide or polypeptide portion of the molecule, or to the carrier may be used, for example as described in WO 99/13719.

Viral or nonviral transfer methods may be used. A number of viruses have been used as gene transfer vectors, including papovaviruses, eg SV40 (Madzak *et al* (1992) *J. Gen. Virol.* **73**, 1533-1536), adenovirus (Berkner (1992) *Curr. Top. Microbiol. Immunol.* **158**, 39-61; Berkner *et al* (1988) *BioTechniques* **6**, 616-629; Gorziglia and Kapikian (1992) *J. Virol.* **66**, 4407-4412; Quantin *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2581-2584; Rosenfeld *et al* (1992) *Cell* **68**, 143-155; Wilkinson *et al* (1992) *Nucleic Acids Res.* **20**, 2233-2239; Stratford-Perricaudet *et al* (1990) *Hum. Gene Ther.* **1**, 241-256), vaccinia virus (Moss (1992) *Curr. Top. Microbiol. Immunol.* **158**, 25-38), adeno-associated virus (Muzyczka (1992) *Curr. Top. Microbiol. Immunol.* **158**, 97-123; Ohi *et al* (1990) *Gene* **89**, 279-282), herpes viruses including HSV and EBV (Margolskee (1992) *Curr. Top. Microbiol. Immunol.* **158**, 67-90; Johnson *et al* (1992) *J. Virol.* **66**, 2952-2965; Fink *et al* (1992) *Hum. Gene Ther.* **3**, 11-19; Breakfield and Geller (1987) *Mol. Neurobiol.* **1**, 337-371; Freese *et al* (1990) *Biochem. Pharmacol.* **40**, 2189-2199), and retroviruses of avian (Brandyopadhyay and

Temin (1984) *Mol. Cell. Biol.* **4**, 749-754; Petropoulos *et al* (1992) *J. Virol.* **66**, 3391-3397), murine (Miller (1992) *Curr. Top. Microbiol. Immunol.* **158**, 1-24; Miller *et al* (1985) *Mol. Cell. Biol.* **5**, 431-437; Sorge *et al* (1984) *Mol. Cell. Biol.* **4**, 1730-1737; Mann and Baltimore (1985) *J. Virol.* **54**, 401-407; Miller *et al* (1988) *J. Virol.* **62**, 4337-4345), and human origin (Shimada *et al* (1991) *J. Clin. Invest.* **88**, 1043-1047; Helseth *et al* (1990) *J. Virol.* **64**, 2416-2420; Page *et al* (1990) *J. Virol.* **64**, 5370-5276; Buchschacher and Panganiban (1992) *J. Virol.* **66**, 2731-2739). To date most human gene therapy protocols have been based on disabled murine retroviruses.

Nonviral gene transfer methods known in the art include chemical techniques such as calcium phosphate coprecipitation (Graham and van der Eb (1973) *Virology* **52**, 456-467; Pellicer *et al* (1980) *Science* **209**, 1414-1422); mechanical techniques, for example microinjection (Anderson *et al* (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5399-5403; Gordon *et al,* 1980; Brinster *et al* (1981) *Cell* **27**, 223-231; Constantini and Lacy (1981) *Nature* **294**, 92-94); membrane fusion-mediated transfer *via* liposomes (Felgner *et al* (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7413-7417; Wang and Huang (1989) *Biochemistry* **28**, 9508-9514; Kaneda *et al* (1989) *J. Biol. Chem.* **264**, 12126-12129; Stewart *et al* (1992) *Hum. Gene Ther.* **3**, 267-275; Nabel *et al,* 1990; Lim *et al* (1992) *Circulation* **83**, 2007-2011); and direct DNA uptake and receptor-mediated DNA transfer (Wolff *et al* (1990) *Science* **247**, 1465-1468; Wu *et al* (1991) *J. Biol. Chem.* **266**, 14338-14342; Zenke *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3655-3659; Wu *et al,* 1989b; Wolff *et al* (1991) *BioTechniques* **11**, 474-485; Wagner *et al,* 1990; Wagner *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4255-4259; Cotten *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4033-4037; Curiel *et al* (1991a) *Proc. Natl. Acad. Sci. USA* **88**, 8850-8854; Curiel *et al* (1991b) *Hum. Gene Ther.* **3**, 147-154). Viral-mediated gene transfer can be combined with direct *in vivo* gene

transfer using liposome delivery, allowing one to direct the viral vectors to the tumour cells and not into the surrounding nondividing cells.

Other suitable systems include the retroviral-adenoviral hybrid system described by Feng *et al* (1997) *Nature Biotechnology* **15**, 866-870, or viral systems with targeting ligands such as suitable single chain Fv fragments.

In an approach which combines biological and physical gene transfer methods, plasmid DNA (or, for example, oligonucleotide/peptide fusion) of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein, and the resulting complex is bound to an adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization, and degradation of the endosome before the coupled DNA is damaged.

Ebbinghaus *et al* (1996) *Gene Ther* **3(4)**, 287-297 describes methods by which TFOs may be delivered to cells using adenovirus-polylysine complexes. Pichon *et al* (2000) *Nucl Acids Res* **28(2)**, describes methods by which the uptake, cytosolic delivery and nuclear accumulation of oligonucleotides may be improved, using histidylated oligolysines.

The "Chariot" system (Active Motif; Morris *et al* (2001) *Nature Biotech* **19**, 1173-1176) may be used. Non-covalent complexes are formed with the molecule to be delivered and the complexes are efficiently internalised into the cell.

Liposome/DNA complexes have been shown to be capable of mediating direct *in vivo* gene transfer. While in standard liposome preparations the gene transfer process is nonspecific, localized *in vivo* uptake and expression

have been reported in tumour deposits, for example, following direct *in situ* administration (Nabel (1992) *Hum. Gene Ther.* 3, 399-410).

Gene transfer techniques which target the molecule directly to a target cell or tissue, is preferred. Receptor-mediated gene transfer, for example, is accomplished by the conjugation of DNA to a protein ligand *via* polylysine. Ligands are chosen on the basis of the presence of the corresponding ligand receptors on the cell surface of the target cell/tissue type. These ligand-DNA conjugates can be injected directly into the blood if desired and are directed to the target tissue where receptor binding and internalization of the DNA-protein complex occurs. To overcome the problem of intracellular destruction of DNA, coinfection with adenovirus can be included to disrupt endosome function.

Preferably, the method of suppressing or modulating the expression of a selected gene is used to suppress (or modulate) expression of a gene in a human cell; in one particularly preferred embodiment the human cell is within a human body.

However, the method of the invention may involve the modification of animal cells (including human cells) outside of the body of an animal (ie an *ex vivo* treatment of the cells) and the so modified cells may be reintroduced into the animal body.

The method of the invention may also involve the *in vitro* investigation or characterisation of modified cells, for example in identifying potential drug targets or screening candidate compounds for potentially pharmaceutically useful activities or properties.

The molecules of the invention, and the methods of the invention, may be used to analyse the role of genes in, amongst other things, chemotherapy resistance or other aspects of tumour development, or resolution of inflammation, or other processes in which apoptosis (including other forms of controlled cell death) is considered to be involved.

A further aspect of the invention provides the use of a molecule of the invention in the manufacture of an agent for suppressing (or modulating) the expression of the selected apoptosis-related gene in a (preferably eukaryotic) cell. It is preferred that the selected gene is an endogenous gene. Other preferences indicated above in relation to earlier aspects of the invention also apply.

It will be appreciated that it is particularly preferred if the molecule is used in the preparation of a medicament for suppressing (or modulating) the expression of a selected apoptosis-related gene in an animal. For the avoidance of doubt, by "animal" we include human and non-human animals.

A further aspect of the invention provides a method of treating a patient in need of suppression (or modulation) of the expression of a selected apoptosis-related gene, the method comprising administering to the patient an effective amount of a molecule of the invention.

Another aspect of the invention provides a method for treating a patient in need of promotion of apoptosis, wherein the patient is treated with a combination method, the method comprising administering to the patient an effective amount of a cell death inducer together with the molecule of the invention. The cell death inducer may be a chemotherapeutic agent or

treatment, for example radiation treatment, as will be well known to those in the field of oncology.

It will be appreciated that suppression of the expression of a selected gene is useful where the expression or overexpression of the selected gene is undesirable and contributes to a disease state in the patient. Examples of undesirable expression of a gene include the expression of certain activated oncogenes in cancer and expression of genes involved in apoptosis inhibition. An increase in expression of the selected gene may be useful where the lack of or insufficient expression of the selected gene is undesirable and contributes to a disease state in the patient. For example, the insufficient expression of a tumour suppressor gene or apoptosis-promoting gene may contribute to cancer; accordingly, it may be useful to increase expression of the tumour suppressor gene or apoptosis-promoting gene.

Suppression of the expression of the Bcl-2 family genes is desirable in the treatment variety of cancers. Similarly, suppression of the expression of other genes, which encode for proteins regulating a programmed cell death, for example Bcl-XI or Akt is desirable in the treatment of cancers.

Further aspects of the invention provides use of a molecule of the invention in the manufacture of a medicament for suppressing (or modulating) the expression of a selected gene in a patient in need of such suppression (or modulation).

Still further aspects of the invention provides a molecule of the invention for use in medicine. Thus, the molecule of the invention is packaged and presented for use in medicine.

Yet still further aspects of the invention provide a pharmaceutical composition comprising a molecule of the invention and a pharmaceutically acceptable carrier.

By "pharmaceutically acceptable" is included that the formulation is sterile and pyrogen free. Suitable pharmaceutical carriers are well known in the art of pharmacy.

The invention will now be described in more detail with reference to the following Figures and Examples wherein:

Figure 1: Schematic representation of fusion molecules and binding to a target site

In A, "1" represents the oligonucleotide module 1 of Example 1; "2" represents the polypeptide module 2. "L" indicates a linker region. In B, D represents an additional delivery peptide. Peptides may be linked to either or both ends of the oligonucleotide; for example, the repressor/modifying polypeptide may be linked to one end and the delivery peptide to the other.

C represents the situation where the oligonucleotide portion has formed a triple helix with double-stranded DNA and the repressor peptide has recruited Sin3-HDAC complex to the site.

Figure 2: Quantitation of Bcl-2 mRNA and the effect of GeneICE on endogenous Bcl-2 mRNA levels

Columns represent the expression of Bcl 2 mRNA after 3 day treatment of BxPC-3 pancreatic cancer cells as indicated, as a ratio relative to the control mRNA value. Each column is a representative of the mean OD and standard

error of the mean of four independent polymerase chain reaction. Mean optical densities were determined.

The TFO secuences for BclP and BclU are (5' to 3'):

BclP GGGTGTGGGGTUTGTGTGTGGT

BclU GGTGTUTTGGTTGGGTGT

L231 is the peptide corresponding to TFO-MAD-NLS and L232 is the peptide corresponding to TFO-NLS-MAD.

Sample treatment	Bcl2/control mRNA ratio
PBS	0.55
L231 peptide	0.49
L232 peptide	0.43
BclP TFO	0.5
BclU TFO	0.6
BclP-L231 50 pmol	0.57
BclP-L231 250 pmol	0.47
BclP-L231 500 pmol	0.35
BclU-L231 50 pmol	0.22
BclU-L231 250 pmol	0.14
BclU-L232 50 pmol	0.29
BclU-L232 250 pmol	0.19
BclU-L232 500 pmol	0.08

Figure 3: Effect of GeneICE bcl-2 on cell viability

PT45 pancreatic cancer cell viability as measured by total mRNA after 3 day treatment as indicated. Columns represent total mRNA μg/ml.

Sample treatment	Total mRNA μg/ml

PBS	571
L231 peptide	302
L232 peptide	294
BclP TFO	117
BclU TFO	155
BclP-L231 50 pmol	181
BclP-L231 250 pmol	39
BclP-L231 500 pmol	44
BclU-L231 50 pmol	173
BclU-L231 250 pmol	43
BclU-L231 500 pmol	25
BclP-L232 50 pmol	191
BclP-L232 250 pmol	89
BclP-L232 500 pmol	49
BclU-L232 50 pmol	211
BclU-L232 250 pmol	147
BclU-L232 500 pmol	127

Figure 4: Effects of unconjugated TFO and BclPL231 conjugate on PT45 cell viability after overnight incubation with 50pmol dose.

Cell number is reduced (ie cell death is induced/promoted), relative to control cells, by the conjugate but not by the unconjugated TFO, particularly with the "Chariot" delivery system (Active Motif; Morris *et al* (2001) *Nature Biotech* **19**, 1173-1176).

Example 1: Construction and use of oligo-regulator peptide fusion molecules.

A series of oligopeptide conjugates useful as gene regulatory molecules has been produced. These consist of at least two specific portions or modules, namely an oligonucleotide capable of forming a DNA triple helix with a selected double-stranded target sequence (Triplex Forming Oligonucleotide, or TFO; Module 1); and a discrete peptide sequence derived from either a gene repressor or activator (Module 2). The TFO is fused to the repressor or activator peptide.

As an example, the TFO is designed to form a triplex with the Bcl-2 promoter region (see accession nos: NM_000657 and NM_0006333). The selected region is very purine rich on one DNA strand and is, therefore, a candidate sequence for forming a DNA triplex by Höogsteen base pairing. The rules for designing potential TFO are summarised in: Vasquez KM and Wilson JH, Trends Biochem Sci, 1: 4-9, 1998. The sequence (5'GGGTGTGGGGTUTGTGTGTGGT3' (BclP) or 5'TUGTGTGGGTGTGGTGUGGG3' or 5'GGTGTUTTGGTTGGGTGT3' (BclU)) was produced as an oligonucleotide (Module 1) with an activated 5' end for chemical coupling to Module 2 peptides. Module 2 peptides explored in this study include human MAD1 transcriptional repressor domain (for example amino acids XXXMNIQMLLEAADYLERREREAEHGYASMLP (where XXX is, for example, a AAA or DDD linker)). The latter is a region known to interact with the histone deacetylase complex protein Sin3a. Additionally, we have explored the use of amino acids XXXMAVESRVTQEEIKKEPEKPIDREKTCPLLLRVF (where XXX is, for example, a AAA or DDD linker) of the human Sap18 protein, also known to associated with Sin3a protein. This region corresponds to a sequence of high evolutionary conservation and overlaps with a region that can mediate gene repression. Module 2 peptides were synthesised in an activated form to enable subsequent coupling to the activated Module 1 oligonucleotide by "native ligation" chemistry (see WO 01/15737 and Stetsenko & Gait (2000) *Organic Chem* **65(16)**, 4900-4908), in which an N-terminal thioester-functionalised peptide is coupled to a 5'-cysteinyl oligonucleotide.

Cell lines for transfection work included prostate cancer LnCap cells and pancreatic cancer PT45 cells, which express endogenous Bcl-2 gene.. Cells were transiently transfected using a standard liposome based transfection method (or alternatively another delivery method, for example electroporation or microinjection) with a varying amount of oligopeptide conjugate. As controls for specificity, cells were also treated with either the oligonucleotide, or unspecific oligopeptide conjugate After suitable times, for example 0.5, 1, 2, 4, 8 12 24 and/or 36 hours, the cell viability was counted using cell counting or other methods described below

The ability of the various oligopeptide conjugates to to reduce cell viability was investigated. Delivery of the increasing concentration of fusion molecule oligo-MAD1 increases the cell death in a concentration dependent manner. After the TFO (ie without a peptide domain) was delivered into the cells in addition to an oligo-peptide fusion molecule, the cell death was less than that seen with the fusion molecule alone, ie equivalent to that seen with a lower concentration of the fusion molecule. This result shows that the molecules with the repressor peptide are more effective regulators of gene activity than the TFO without a peptide domain, and the TFO may compete with the oligo-peptide fusion molecule for binding to the target sequence. The oligo and unspecific conjugate molecule had no cell killing effect, demonstrating the specificity of the oligopeptide conjugates in Bcl-2 controlled cell viability.

This example demonstrates the design and construction of fusion molecules consisting of DNA binding oligonucleotides and functional peptides, and their delivery into the cells. The oligopeptide conjugates are able to induce a specific biological response in a targeted manner. Thus, oligopeptide conjugates can be designed to be potent regulators of biological activity.

Example 2: Repression of chromosomal genes by oligo-regulator fusion molecules

Fusion molecules are able to regulate gene activity, when such genes are integrated into the genome. Fusion molecules containing a DNA binding oligonucleotide (TFO) fused to a MAD1, were designed and constructed as described in Example 1.

The fusion molecules were delivered into the cells and experiments were carried out to measure Bcl-2 gene activity. .

The ability of the various oligopeptide conjugates to repress Bcl-2 gene activity was investigated. Delivery of the increasing concentration of fusion molecule oligo-MAD1 suppressed the Bcl-2 gene actity, as measured by mRNA, in a concentration dependent manner. After the TFO (ie without a peptide domain) was delivered into the cells in addition to an oligo-peptide fusion molecule, the gene repression was less than that seen with the fusion molecule alone, ie equivalent to that seen with a lower concentration of the fusion molecule. This result shows that the molecules with the repressor peptide are more effective regulators of gene activity than the TFO without a peptide domain. The oligo and unspecific conjugate molecule had no cell killing-effect, demonstrating the specificity of the oligopeptide conjugates in gene regulation.

Furthermore, the repression was measured at different times in order to establish a time course for repressor effects. The fusion molecules were more effective repressors that TFOs alone. The effect was also specific (for example, the unfused oligonucleotide and unspecific molecules did not have

the same effect as the oligo-peptide fusion). In addition, the repression by fusion molecules was seen at later time points than any repression seen by the TFO alone, suggesting a more permanent effect.

Thus, the fusion molecules described in example 1 are able to regulate chromosomal gene activity. Fusion molecules with a DNA binding oligonucleotide targeting portion are able to target specific chromosomal genes. A Gene ICE repressor peptide fused to the DNA binding oligonucleotide is able to repress predetermined chromosomal gene activity. Thus, the described fusion molecules are potent regulators of chromosomal gene activity.

Endogenous gene regulation is measured, for example by assessing transcription of the gene (for example using PCR) or by assessing the quantity or activity of the encoded polypeptide. In an example, the oligonucleotide is directed to the Bcl-2 gene regulatory site. Examples of suitable RT-PCR primers include

5' TCCGGTATTCGCAGAAGTCC 3'
5' ATCAGAAGAGGATTCCTGCC 3'
(used to assess BclP) and

5' TGATGGAGCTCAGAATTCC 3'
5' TGCCTCTCCTCACGTTCC 3'
(used to assess BclU).

In an example, the prostate cancer cell line LnCap and pancreatic cancer cell line PT45 are treated with the oligonucleotide-peptide fusion comprising a MAD1 described in Example 1. Transfected cells are optionally identified and/or isolated (for example using a GFP marker and FACS techniques) and are assayed for Bcl-2 gene expression.

For example, the cells were transfected with GeneICE Bcl-2. After 12, 24, 48 and 72 hours, the mRNA levels were measured by reverse transcriptase PCR.

Example 3: Fusion molecule binding to a target sequence and histone deacetylase complex.

This Example demonstrates that the fusion molecule binds to a specific target sequence as well as to a component of histone deacetylase complex. The fusion molecules containing an oligonucleotide (TFO) and a repressor peptide were produced as described in Example 1.

The different fusion molecules were incubated with labelled oligonucleotide, which was made complementary to the oligo part of a fusion. The same fusion molecules were also incubated with Sin3, which is a component of a histone deacetylation complex. Furthermore, the fusions were also incubated with both the complementary oligonucleotide and Sin3 protein.

The complexes were then analysed by standard band shift analysis methods. The fusion molecules were able to bind to both the labelled complementary oligonucleotide as well as the Sin3 protein, both separately and simultaneously. The unspecific fusions were not able bind the labelled oligo or Sin3, thus demonstrating the specificity of the effect with repressor fusions.

It can be concluded that the repressor fusions can specifically bind their target sequences. The repressor fusions are able to recruit histone

deacetylase complexes by binding proteins that are part of this complex, and by binding their target sequences and recruiting the histone deacetylase complexes simultaneously, the described fusion molecules are very potent and specific repressors of gene activity.

Example 4: Target validation protocol

The available DNA sequence for the gene of interest (including flanking sequence) is analysed in order to select a suitable site for targeting an oligo/peptide to. The oligo/peptide is synthesised and may be tested prior to use in the intended cells or animals or humans, for example using a reporter gene system. The oligo/peptide may be used or tested further in cells *in vitro* or in animals or humans.

Once a gene sequence has been provided, the process will involve:
The gene of interest (including flanking sequences if necessary) will be scanned for unique sequence elements not found elsewhere in the human genome using bio-informatics data-mining tools (for example the Genetics Computer Group (GCG) program as used in Perkins *et al* (1998) *Biochemistry* **37**, 11315-11322). A nucleic acid based DNA binding molecule predicted to bind to the identified unique sequence (for example as a TFO) is designed and synthesised.

The DNA binding molecule is likely to be an oligonucleotide, preferably with the following features:
(a) at least 16 nucleotides in length
(b) targeted to a gene promoter or at or near to the transcription initiation site of the gene.

It is preferred that the target site for the binding to a TFO is purine-rich in one strand.

The TFO may be pyrimidine rich (predominantly C or T); purine rich (predominantly G or A) or mixed (predominantly G or T, or G, A or T). CT TFOs are considered to bind in a parallel motif, in which the third strand (TFO) has the same 5' to 3' orientation as the purine strand of the duplex. GA TFOs are considered to bind in an antiparallel motif, in which the TFO is oriented oppositely to the purine strand. Mixed TFOs may bind in a parallel or antiparallel motif, depending on the target sequence. Base pairing arises from formation of Hoogsteen hydrogen bonds in parallel triplexes (T:AT, C^+:GC and G:GC) and reverse Hoogsteen hydrogen bonds in antiparallel triplexes (G:GC, A:AT and T:AT).

It is intended that the oligonucleotide is a DNA oligonucleotide, possibly with stabilising chemical modifications. Alternative bases, for example N^6-methyl-8-oxo-2-deoxyadenine may be used in place of cytosine, 2-deoxy-6-thioguanine in place of guanine or 7-deaza-2-deoxyxanthine in place of thymine.

The repressor peptide or peptides may be produced in bulk using a peptide synthesiser and stored frozen until used.

The repressor peptide-DNA binding molecule construct is prepared and purified. The chemistry used may be that described in WO 01/15737. Kits are available from Link Technologies.

The construct may be quality controlled by mass spectroscopy and/or by use of labelled complementary oligonucleotide or labelled antibody moieties

(using for example fluorescent, chemiluminescent or enzyme labels). Typically in such a method the construct is added to a solid support on which an antibody that binds to the peptide portion of the construct is immobilised and a labelled oligonucleotide that binds to the oligonucleotide portion of the construct is added. In this method detection of the label bound to the solid support demonstrates that the construct is intact. In another typical format the oligonucleotide may be attached to a solid support and the antibody labelled.

A reporter gene construct may be prepared for the gene of interest (though this is not generally necessary).

- The candidate DNA binding oligonucleotide or oligo/peptide may be tested for the following:
    - Affinity of binding to the target sequence;
    - Specificity of binding by exposure to a whole genome DNA chip.
- The oligo/peptide may be tested for effectiveness using the reporter gene system.

The oligo/peptide may then be used for modulating or suppressing expression of the gene of interest in the cell or animal of interest.

Example 5: Target validation

The oligo/peptide fusion molecules may be used to validate apoptosis-related drug targets. This will involve:

- Carrying out the protocol set out in Example 1.

- Delivery of the construct into cells or tissues. These may be normal or disease tissues, cell lines or primary cells appropriate to the study of the molecule of interest.
- Analysis of the phenotype by any expression analysis methods; or any functional analysis such as assessment of cell motility, growth or apoptosis analysis.
- Comparison with any available data for a particular disease and analysis of desired effects such as cell death or motility.

The obtained data will be used to validate the pre-determined drug targets for drug development programmes.

Example 6: Patient treatment example

A oligo/peptide fusion is produced as described which targets an apoptosis-related gene, for example Bcl-2. The fusion molecules are prepared in a sterile environment and formulated into liposomes. The fusion-containing liposomes are targeted into the vicinity of a tumour, for example breast or prostate. The liposomes are taken up by cancer cells and apoptosis is promoted selectively in respective cells.

Example 7: Target identification screen

The oligo/peptide fusion molecules will be used to identify apoptosis-related drug targets. This will involve:

- Preparation of a fusion molecule as set out in Example 1
- Delivery of the fusion molecule into the cells or tissues. These may be normal or disease tissues, cell lines or primary cells.

- Analysis of gene expression profile resulting from gene silencing, using DNA arrays. This indicates the effect of the construct on overall gene expression in the model.
- Analysis of the phenotype by any expression analysis methods, or any functional analysis such as cell motility, growth or apoptosis analysis.

The obtained data will be used to find potential drug targets for diseases such as breast or prostate cancer. These targets can be further validated by appropriate methods including any further similar screens, *in vitro* methods and cell and animal models.

Example 8: Down-regulation of gene expression by oligo/peptide fusion molecules is associated with chromatin histone deacetylation

As seen above the oligo/peptide fusion molecules are effective in repressing gene expression. We propose that this is due to a MAD1-mediated change in the histone acetylation state of DNA at or close to where the oligo binds.

To show that reduced gene expression is associated with chromatin histone deacetylation, the Chromatin immunoprecipitation (ChIP) method may be used. Chromatin immunoprecipitation is carried out using a ChIP Assay kit according to the manufacturer's instructions (Upstate Biotechnology, Bucks, UK).

PT45 or BxPC-3 pancreatic cancer cells are grown and propagated and incubated with oligo/peptide fusion molecules which are effective in repressing apoptosis-related gene expression. Untreated cells are used as a control.

Cells are grown to 95% confluence on 35cm tissue culture plates in DMEM, lacking phenol red, supplemented with 5% DSS, P/S/G and G418 (100μg/ml). Hygromycin B (80μg/ml) and doxycycline (1μg/ml) are added as appropriate. 30 minutes prior to fixation, E2 (10-8M) or ethanol (as a control) is added to the cells. 37% formaldehyde is added dropwise directly to the medium to a final concentration of 1%. Cells are incubated for 10 minutes at 37°C.

On ice, the medium is aspirated from the plates, cells are washed twice with ice cold PBS containing 1x protease inhibitors (PI) (Sigma, Dorset, UK). For harvesting, 1ml of ice cold PBS containing 1x PI is added to the plate and the cells scraped into pre-cooled microfuge tubes, using a rubber policeman. Cells are pelleted by centrifugation at 2000rpm for 4 minutes, at 4°C. The pellets are resuspended in 400μl of warmed ChIP SDS-lysis buffer (1% SDS; 10mM Na EDTA pH 8.0; 50mM Tris-HCl pH 8.1) containing PI, and incubated on ice for 10 minutes.

The lysates are sonicated to shear the DNA into 200 – 1000bp lengths. During sonication, the samples are placed in an ice-water beaker, to keep them cold in order to prevent sample degradation. Sonication is carried out using a Soniprep 150 sonicator with attached Soniprep 150 exponential titanium probe (Sanyo-Gallenkamp, Leics, UK) with four 10 second bursts, separated by 30 second intervals.

Samples are centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant is collected into 15ml sterile falcon tubes and diluted 10 fold with 3600μl ChIP dilution buffer (0.01% SDS; 1.1% Triton-X-100; 1.2mM Na EDTA pH 8.0; 16.7mM Tris-HCl, pH 8.1; 167mM NaCl). The samples are then divided into two 2ml aliquots in 2.5ml tubes, one for incubation

with an anti-acetylated histone H4 antibody, ChIPs grade (Upstate Biotechnology, Bucks, UK), and the other for use as a no antibody control.

To reduce non-specific background, each 2ml aliquot is pre-cleared by adding 80µl of salmon sperm DNA/protein A agarose-50% slurry (suspended in 10mM Tris-HCl pH 8.0; 1mM Na EDTA pH 8.0) for 30 minutes at 4°C on a vertical rotating platform (Stuart Scientific, Staffs, UK). The agarose beads are then pelleted by a 30-second centrifugation at 1000 rpm and the supernatant fractions collected into fresh 2.5ml tubes. The immunoprecipitating antibody is added at a dilution of 1:500 to the first sample but not to the no antibody control sample. Both tubes are incubated overnight at 4°C on a vertical rotating platform (Stuart Scientific, Staffs, UK).

60µl of salmon sperm DNA/protein A Agarose-50% slurry are incubated with each tube for 1 hour at 4°C, with rotation, to collect the antibody/histone complexes or non-specifically bound proteins in the case of the no antibody control. The agarose beads are pelleted by brief centrifugation at 800 rpm for 1 minute. The supernatants are carefully transferred into fresh 2.5ml tubes and stored at –20°C.

The protein A Agarose beads/antibody/histone complex is washed for 5 minutes on a vertical rotating platform at 4°C with 1ml of each of the following buffers in the order listed below:

(a) Low Salt Immune Complex Wash Buffer - **one wash**
(0.1% SDS; 1% Triton-X-100; 2mM Na EDTA pH 8.0; 20mM Tris-HCl pH 8.1; 150mM NaCl)

(b) High Salt Immune Complex Wash Buffer - **one wash**

(0.1% SDS; 1% Triton-X-100; 2mM Na EDTA pH 8.0; 20mM Tris-HCl pH 8.1; 500mM NaCl)

(c) LiCl Immune Complex Wash Buffer **- one wash**

(0.25M LiCl; 1% NP40 (nonidet); 1% deoxycholate; 1mM Na EDTA pH 8.0; 10mM Tris-HCl pH8.1)

(d) 1x TE **- two washes**

(10mM Tris-HCl, 1mM Na EDTA pH 8.0)

The histone/immune complex is eluted from the agarose beads by addition of 250µl freshly prepared Elution buffer (1% SDS; 0.1M $NaHCO_3$). The samples are vortexed briefly to mix and incubated at room temperature for 15 minutes on a vertical rotating platform. The beads are centrifuged at 1000 rpm for 2 minutes at room temperature and the eluate transferred to fresh microfuge tubes. The elution step is repeated with a further 250µl of Elution buffer and the eluates combined.

20µl of 5M NaCl are added to the eluates and histone-DNA crosslinks reversed by heating to 65°C for at least 4 hours. 10µl of 0.5M Na EDTA pH 8.0, 20µl of 1M Tris-HCl, pH 6.5 and 2µl of 10mg/ml Proteinase K are added to the eluted samples. The crosslinks are also reversed on the supernatant fraction from the IP. 40µl of 0.5M Na EDTA pH 8.0, 80µl of 1M Tris-HCl, pH 6.5 and 8µl of 10mg/ml Proteinase K are added to supernatant samples. Samples are incubated for 1 hour at 45°C to degrade protein in the samples.

20µg of glycogen are added to the samples as an inert carrier and then the sample DNAs are recovered by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. DNA pellets are resuspended in 50µl sterile water for PCR reactions. 1µl of sample and 35 – 40 cycles are used

for each PCR-amplification. Computer-based image analysis (NIH image analysis program) is employed to evaluate the relative levels of the Bcl2 PCR product, compared with the ß-actin and no antibody controls. This allowed a calculation of the relative amount of the gene transcript contained within a sample to be compared with that in other samples.

An example of the type of result that may be achieved is shown in Figure 6.

The amount of precipitated Bcl-2 DNA, and hence the degree of acetylation of the chromatin histone proteins associated with the Bcl2, may be decreased by approximately 75% in the cells in which Bcl2 gene expression is inhibited by the described method compared to untreated cells.

Example 9: Gel shift assay

The previous examples have demonstrated that oligo/peptide fusion molecules are able to regulate targeted gene activity. This is considered to be due to a MAD1-mediated change in the histone acetylation state of DNA at or close to where the oligo binds.

A gel shift assay is used to demonstrate that the oligo/peptide fusion molecules can bind to DNA fragment containing the target promoter.

A DNA fragment containing the target gene sequence is incubated with the oligonucleotide or oligo/peptide fusion. Samples are subjected to non-denaturing gel electrophoresis in order to characterize triplex-mediated photoadduct. Adducts are detected in samples containing the oligonucleotide or oligo/peptide fusion which shifted with increasing doses.

In this way it is possible to show that oligo/peptide fusion molecules can bind to DNA fragment containing the target promoter.

Example 10: Oligo/peptide fusion molecules with a nuclear localisation signal.

The peptide portion of the molecule may have a nuclear localisation signal (NLS) to target the molecule to the nucleus. The peptides used in this example are:

a) DDD-MAD1-DDD-NLS,

which has the amino acid sequence:

(Link)DDDMNIQMLLEAADYLERREREAEHGYASMLPDDDPKKKRK V (carboxamide)

and,

b) DDD-NLS-DDD-MAD1,

which has the amino acid sequence:

(Link)DDDPKKKRKVDDDMNIQMLLEAADYLERREREAEHGYASML P (carboxamide)

The NLS is a 7 amino acid (sequence PKKKRKV) functional nuclear localisation signal derived from the SV40 T-antigen.

The DDD linker sequence and 29 amino acid MAD1 amino acid sequence are the same as those discussed in the earlier examples.

To demonstrate that the DDD-MAD1-DDD-NLS, and DDD-NLS-DDD-MAD1 peptide sequences are targeted to the nucleus, PT45 human pancreatic cancer cells were transfected using with GeneICE oligopeptides consisting of both the DDD-MAD1-DDD-NLS, and DDD-NLS-DDD-MAD1 peptide sequences linked to a Cy3 labelled oligonucleotide by standard Lipofectamine 2000 protocols. The cells were then fixed in formaldehyde and stained with DAPI nuclear stain using the manufacturer's recommended procedure. Examination of the cells by fluorescence microscopy showed that the Cy3 labelled GeneICE oligopeptide molecule was co-localised with the nuclear DAPI stain. From the resulting experimental data it was concluded that the NLS is very effective at targeting the peptide to the nucleus.

The DDD-MAD1-DDD-NLS, and DDD-NLS-DDD-MAD1 peptide sequences may mediate target gene expression when incorporated into an oligo/peptide molecules of the invention. This can be demonstrated using the experimental approach outlined in Examples 8 and 9.

For example, DDD-MAD1-DDD-NLS, and DDD-NLS-DDD-MAD1 peptide sequences can be incorporated into the oligo/peptide molecules:

Bcl2:-DDD-MAD1-DDD-NLS, and
Bcl2:-DDD-NLS-DDD-MAD1.

Bcl2 is the TFO oligo sequence shown in Example 8 (5'GGGTGTGGGGTUTGTGTGTGGT3' or

5'TUGTGTGGGTGTGGTGUGGG3' or

5'GGTGTUTTGGTTGGGTGT3').

The Bcl2:-DDD-MAD1-DDD-NLS and Bcl2:-DDD-NLS-DDD-MAD1 molecules are then transfected into test cells, for example LNCap cells. Using the experimental procedures outlined above it is possible to show the effect of the Bcl2:-DDD-MAD1-DDD-NLS and Bcl2:-DDD-NLS-DDD-MAD1 molecules on target gene expression.

Example 11: assessment of constructs for modulating apoptosis-related gene expression

Binding:

The binding of both the TFOs and the GeneICE™ molecules to their target DNA sequences is assessed by use of a gel shift assay. To do this, a know quantity of TFO is incubated in TFO binding buffer (20mM TRIS (pH 7.6), 10% v/v Glycerol, 10mM $MgCl_2$) for 1 hour at 37°C with a trace quantity of ^{32}P labelled target DNA, previously amplified from genomic DNA using PCR and end-labelled using T4 Kinase. The reaction is loaded on to a 12% non-denaturing polyacrylamide gel using a non-denaturing loading buffer (10X stock concentrations: 250mM TRIS (pH 7.5), 40% v/v Glycerol, 10mM $MgCl_2$, 0.2% w/v Bromophenol Blue) and electrophoresed in a TRIS-Borate-Magnesium running buffer (0.5X TBE, 10mM $MgCl_2$) for 3-4 hours. The gel is dried before being exposed to autoradiography film for visualisation of the bands.

Three different sized bands are expected on the gel. Where the concentration of TFO or construct is low only the small DNA band is seen. When the concentration of TFO rises to allow formation of triplex DNA a middle sized triplex band appears and when the concentration of the GeneICE™ molecules rises to allow formation of triplex DNA a larger triplex band is seen. The Kd of the TFO or GeneICE™ molecule is determined by the concentration where there are equal amounts of duplex DNA band and triplex DNA band. Kd values in the range of 10^{-6} to 10^{-9}M are expected, with the Kd for the GeneICE™ molecule being up to 1 log higher than for the TFO.

mRNA Expression:

mRNA expression may be checked by two methods. Initially, total RNA will be extracted from treated cells and used for either RT-PCR or Northern blot analysis. In RT-PCR analysis, the mRNA is reverse transcribed into cDNA by use of reverse transcriptase with oligo dT primers. This cDNA is used for a semi-quantitative PCR using primers designed to amplify Bcl-2 coding sequence. β-actin PCR is also be carried out and the relative amount is used to quantify the down regulation seen with GeneICE™ treatment. Results obtained from RT-PCR analysis is confirmed by Northern blot analysis. Total RNA extracted from treated cells is electrophoresed on a TBE gel and transferred to a nylon membrane. A single stranded DNA probe, previously labelled with ^{32}P by PCR is used to hybridise to the Bcl-2 mRNA sequence. Non-specific binding is eliminated by stringent washing of the membrane and autoradiography is used to visualise the amount of mRNA present. RNA loading is controlled for by using a probe for β-actin mRNA.

The mRNA expression experiments show specific down regulation of the Bcl-2 gene after treatment with Bcl-2 targeted GeneICE™ molecules. An intermediate level of down regulation arises by treatment with TFO only or TFO linked to delivery peptide. The use of deacetylase inhibitors, such as TSA, reverses the down regulation due to the GeneICE™ molecule but not the TFO mediated down regulation of gene expression. These experiments also show both a dose dependant effect and a time dependant effect of the GeneICE™ construct, with an increase in dose showing more down regulation until the down regulation becomes non-specific. The dose dependant effect will show a prolonged down regulation from a single dose that peaks after a matter of hours and lasts for several days.

Protein Expression:

Protein expression is assessed by immuno-blot analysis. Cells are lysed on ice in an SDS containing lysis buffer in the presence of protease inhibitors. The cell lysate is cleared by centrifugation and the amounts of protein are determined by use of protein assay. An equal amount of protein from each sample is loaded into the wells of a 15% SDS-polyacrylamide gel and electrophoresed at 150V for 2 hours using a 25mM TRIS, 192mM glycine, 0.1% w/v SDS running buffer. After separation the protein is transferred onto a PVDF membrane by semi-dry electrophoretic transfer at 5.5mA/cm^2 using a 48mM TRIS, 39mM glycine, 0.0375% w/v SDS, 20% v/v methanol transfer buffer. Non-specific antibody binding is blocked by incubation in 5% w/v fat-free milk in PBS/0.1% w/v Tween 20 (PBST). The membrane is incubated with an anti-Bcl-2 primary antibody at a 1:2000 dilution in 2% milk for 1 hour at room temperature. Excess antibody is washed off using 2% milk and a horse radish peroxidase linked secondary antibody is used at 1:10000 in 2% milk. Excess antibody is removed using two washes in PBST followed by a single wash in PBS. Protein is detected by use of enhanced chemi-luminesence and autoradiography. Loading is corrected for by probing for actin using the same procedure but using an anti-actin primary antibody at 1:500 dilution in place of the anti-Bcl-2 antibody.

Protein expression is confirmed by use of a Bcl-2 ELISA kit following the instructions provided by the manufacturer.

Similar experiments as with mRNA expression are carried out for protein analysis. A time and dose dependant down regulation of protein expression after treatment with GeneICE™ constructs is expected, which follows that

seen in the mRNA expression profile. Protein levels drop approximately 15-20 hours after the mRNA levels and remain low for several days. Deacetylase inhibitors prevent this specific Bcl-2 protein down regulation whilst treatment with TFO presents an intermediary phenotype.

Cell Death:

The reduction in protein expression may, in some cell lines, be sufficient to cause cell death by apoptosis. If this is the case, an expression plasmid with Bcl-2 under the control of a non-endogenous promoter (such as the SV40 promoter) is transfected into the cell line and levels of endogenous mRNA are assessed as described using primers specific to the endogenous gene.

Cell death is assessed by trypan blue staining of cells and counting using a counting chamber. Cells are treated with GeneICE™ constructs for an appropriate length of time and trypsinised from the well using a known volume of trypsin. 20µl of the cell suspension are mixed with 20µl of trypan blue and loaded into a counting chamber. Between 30 and 300 cells are counted using the grids of the counting chamber as a guide to volume to give counts/ml and viability of the cells is assessed by trypan blue staining indicating the non-viable cells.

MTT assays are used to confirm the data provided by cell counts. Experiments are established in 96 well plates and after treatment MTT is added to the wells for up to 1 hour. Medium is aspirated and the cells washed. The precipitate remaining is dissolved in DMSO and the absorbance is read at 520nm.

GeneICE™ treatment leads to a reduction in the number of cells in the culture and a decrease in the viability of the culture. The use of deacetylase inhibitors reverses these effects as will inhibitors of apoptosis. TFO controls present an intermediate phenotype.

Apoptosis:

The method of the observed cell death is investigated by use of a Terminal deoxytransferase-mediated dUTP nick-end labelling (TUNEL) kit following the instructions provided by the manufacturer. The experiment is carried out over a range of different time points to determine the point at which most dying cells are seen. The cell death seen after treatment with GeneICE™ is expected to be via an apoptotic mechanism. An inducer of apoptosis is used as a positive control to compare the cells to whilst apoptosis inhibitors are able to prevent the cell death and hence the appearance of apoptotic cells in the TUNEL assay. Deacetylase inhibitors also prevent apoptosis from occurring in GeneICE™ treated cultures.